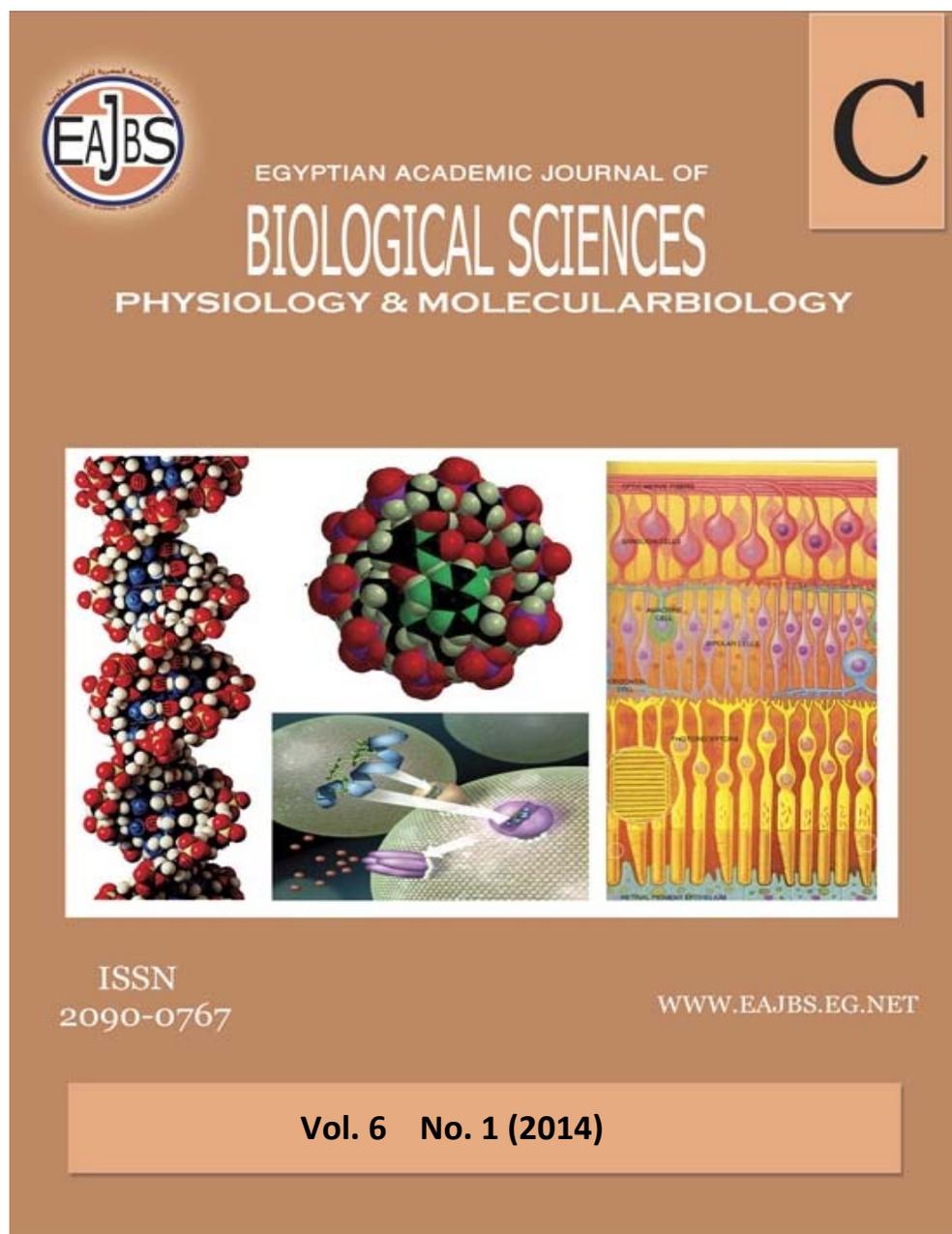


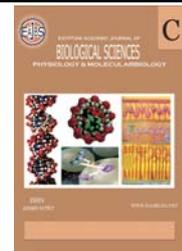
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## Protective effect of Mulberry Leaf Extract on male rat fertility affected with Methotrexate-cytotoxicity

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### ABSTRACT

Methotrexate is widely used in treatment of cancer, psoriasis, and rheumatic diseases but it can be induce reproductive toxicity. Mulberries have been reported to be important antioxidant. Therefore, the present study aimed to investigate the protective effects of ethanolic leaf Mulberry extract against reproductive toxicity of Methotrexate in male rats. Twenty male albino rats were divided into four equal groups (n=5), the first served as negative control, the second received mulberry extract (100 mg/kg b.wt), the third group received Methotrexate (20mg/kg) the fourth received Methotrexate after one week of treatment with mulberry extract (100 mg/kg b.wt.). Treatment was continued for 4 weeks. Results of the study displayed that Methotrexate caused a significantly decrease in Gonadisomatic index weights, testosterone, LH and FSH in serum as compared with normal control. Mulberry extract protection against MTX-induced reproductive injury was reflected by significantly increased serum testosterone, LH and FSH levels compared to those in MTX treated Rats ( $P < 0.05$ ). Histological observations also revealed that damages within the seminiferous tubules and vascular degeneration of the germ cells and Sertoli cells cytoplasm were observed apparent in rats after MTX administration. In contrast, the damage was markedly attenuated in the mulberry treated group. In conclusion, treatment with mulberry leaf extract alleviates Methotrexate-associated hazards and protects the testicular tissues.

### INTRODUCTION

Of all cases of infertility in humans, 20% are due entirely to a male factor, with an additional 30-40% involving both male and female factors (El-Beshbishy *et al.*, 2006). Methotrexate, an immunosuppressive drug used to treat cancer, psoriasis, and rheumatic diseases, is a folic acid antagonist that binds to the enzyme dihydrofolate reductase.

This inhibits synthesis of thymidylate, serine, and methionine, which disrupts synthesis of DNA, RNA, and protein and leads to cell death (Cronstein, 2005; Martínez Lopez, *et al.*, 2009). In this context French *et al.*, 2003 reported that methotrexate's effect on men's fertility where Methotrexate damages or kills cells undergoing division, a process continually occurring during production of spermatozoa.

The acceptance of the use of natural products regarding the safety and cost of conventional medicines, are among the factors contributing to the increasing consumption of herbal medicines and developing pharmaceutical with potential efficacy and safety (Marques, 1992; Petrovick *et al.*, 1997).

Mulberries (*Morus alba* L.) are widely distributed in Asia where they have been used as a traditional food and found to possess pharmacological benefits such as liver protection, eyesight improvement, blood pressure reduction, and helping to prevent cardiovascular disease. Dietary mulberry (as fruit) has been reported to have antihyperlipidemic (Beshbishy *et al.*, 2006). Antidiabetic (Kimura *et al.*, 2007) and antioxidative effects (Andallu *et al.*, 2001; Isabelle *et al.*, 2008). These properties are due to mulberry's constituents, including high levels of anthocyanins and polyphenolics, which have many biochemical activities, including antioxidant activities. Antioxidants are the main defense against oxidative stress induced by free radicals. There are preventive antioxidants and scavenger antioxidants. Preventive antioxidants, e.g. metal chelates and metal-binding proteins, block the formation of new free radicals, whereas scavenger antioxidants remove the free radicals that have already formed (Sanocka and Kurpisz, 2004).

In this context, we evaluated the effect of methotrexate toxicity on male rat fertility and administered mulberry

extract as a protective agent to assess any change in the effects of MTX intoxication.

## MATERIALS AND METHODS

### Experimental design:

Twenty adult male albino rats were used in the present study. They were divided into four main groups each of 5 rats: Group I was kept without treatment and served as control. Group II was given oral gavage injections of mulberry extract at a dosage of 100 mg/Kg twice weekly for total durations of 4 weeks. Group III the rats were injected I.P with MTX (Methotrexate hydrate, Sigma) in a single dose of 20 mg/kg (Jahovic *et al.*, 2003). Group IV injected with MTX (20mg/kg) after one week of mulberry extract treatment then rats treated with mulberry extract for total durations of 4 weeks.

### Sample collection and calculating the gonadosomatic indices (GI):

Each animal was weighed alive and sacrificed after anaesthesia with chloroform in a close container. The abdominal part was then dissected open through a mid ventral abdominal incision. The ischiatic arch was completely removed to expose the reproductive organs. The testis was collected and weighed. The gonado-somatic index by weight was calculated by dividing the average of the weights of the right and left testicles by the body weight (g).

### Estimation of serum FSH, LH and testosterone levels

At the end of the experimental period a blood sample was drawn with a syringe from each rat's heart, and analyzed for serum FSH, LH and testosterone levels using an ELISA technique. As all rats had been assigned randomly to the groups at the beginning of study, serum levels in the control group were referred to as the normal range and levels in the other groups were compared with treated group.

### Histopathology

Small slices of testes were taken and fixed in 10% formalin for 24 hours, and were imbedded in paraffin. Five-micron-thick sections were routinely stained with hematoxyline and eosin.

### Statistical analysis

Data were given as mean  $\pm$  SE and percentage. Statistical comparisons between different groups were done using one-way analysis of variance (ANOVA) followed by the Duncan multiple comparison test (Field, 2000), to judge the difference between various

groups. Significance was accepted at  $\leq 0.05$ .

## RESULTS

### Gonadisomatic index

The results presented in Fig. 1 showed MTX-treated rats showed significant decrease in GSI as compared with normal control group. While, oral administration of alcoholic extract of mulberry leaves at dose 100 mg/kg body weight (group II) for 4 weeks to MTX-treated rats had significant increased in GSI ( $P < 0.05$ ) as compared with MTX-treated (group IV).

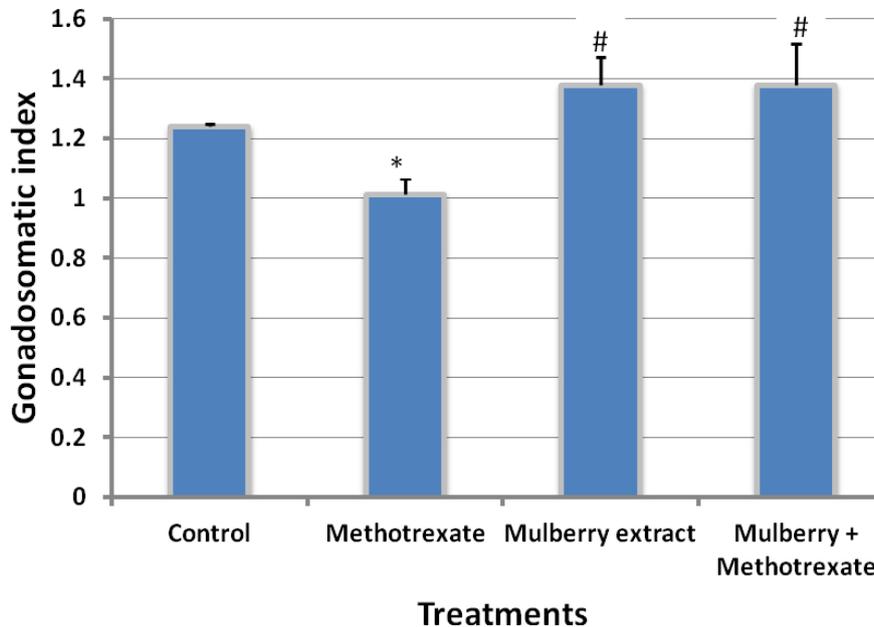


Fig. 1: Effect of methotrexate (MTX) alone or in combination with mulberry extract on Gonadosomatic index of adult male rats. Values are mean  $\pm$  SE. \* and # indicate significant change from normal control and MTX, respectively, at  $p < 0.05$  using ANOVA followed by Duncan as a post ANOVA test.

### Hormones

Methotrexate at doses of 20 mg/kg significantly reduced FSH, testosterone and LH compared to normal control. Administration of 100 mg/kg

mulberry extract to MTX-treated animals significantly elevated FSH, testosterone and LH compared to MTX alone (Figs. 2, 3 and 4 respectively).

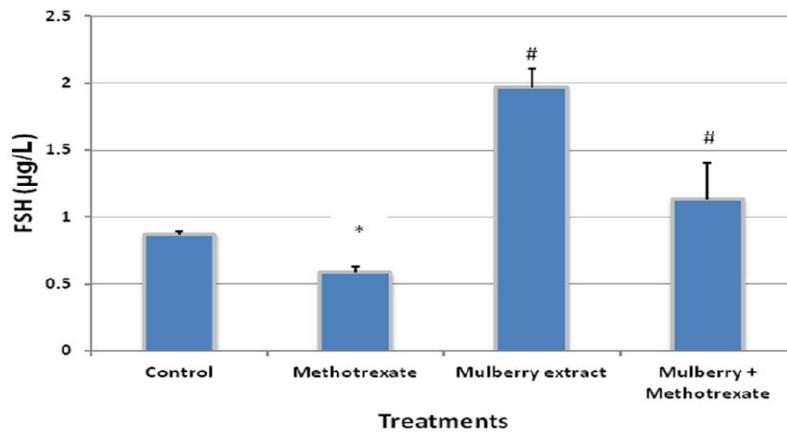


Fig. 2: Effect of methotrexate (MTX) alone or in combination with mulberry extract on FSH of adult male rats. Values are mean  $\pm$ SE. \*and # indicate significant change from normal control and MTX, respectively, at  $p < 0.05$  using ANOVA followed by Duncan as a post ANOVA test.

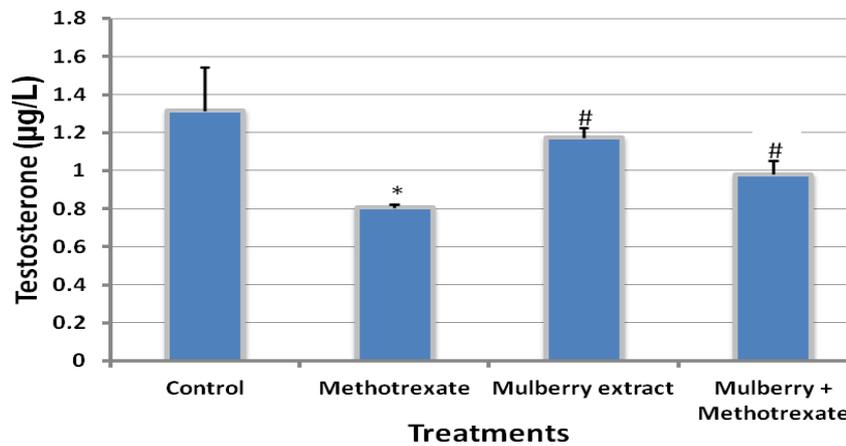


Fig. 3: Effect of methotrexate (MTX) alone or in combination with mulberry extract on testosterone of adult male rats. Values are mean  $\pm$ SE. \*and # indicate significant change from normal control and MTX, respectively, at  $p < 0.05$  using ANOVA followed by Duncan as a post ANOVA test.

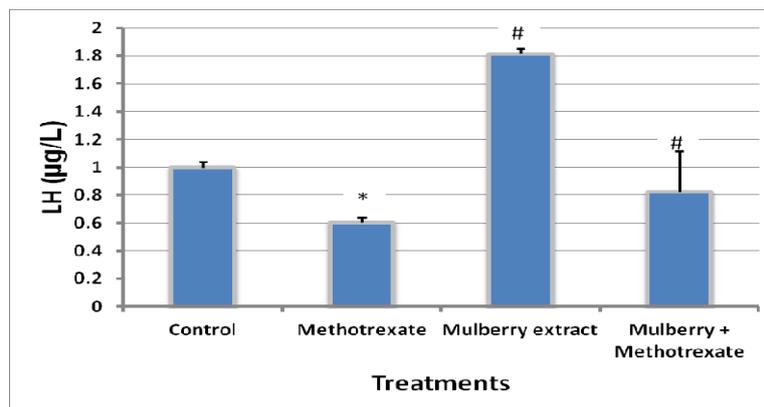


Fig. 4: Effect of methotrexate (MTX) alone or in combination with mulberry extract on LH of adult male rats. Values are mean  $\pm$ SE. \*and # indicate significant change from normal control and MTX, respectively, at  $p < 0.05$  using ANOVA followed by Duncan as a post ANOVA test.

**Histopathological results.**

The normal architecture of testicular seminiferous tubules and interstitial spaces were shown in the control rats (Fig. 5A). The testes of rats, intoxicated with MTX alone showed an extensive degeneration in spermatids and spermatozoa, blood vesesels congestion

and interstitial fibrosis (Fig 5B and 5C). Mulberry treated rats showed normal histological structure of most seminiferous tubules (Fig. 5D). MTX-treated rat given mulberry extract at 100 mg.kg b.wt., for 4 weeks showing normal histological structure of most seminiferous tubules (Fig. 5E).

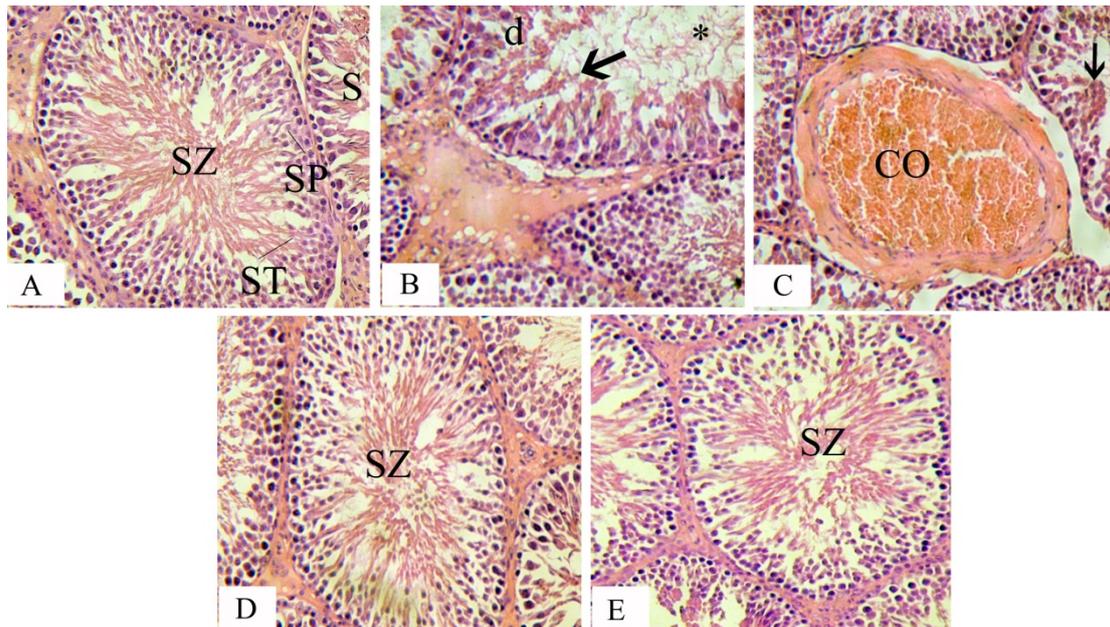


Fig. A: Testis of a control rat showing normal histological structure of active mature functioning seminiferous tubules(S) associated with complete spermatogenic series. The peripheral layer of cells is composed of spermatocytes (SP) followed by a zone of spermatids (ST) and finally spermatozoa (SZ) about to be released into the lumen. (H&E, X200)

Fig. B: Testis of a MTX-treated rat showing marked degeneration (d) of most seminiferous tubules with degeneration of spermatogenic series in tubular lumen and interstitial fibrosis. The arrow indicate mild disintegration of the seminiferous tubules with loss of spermatids and spermatozoa (astriks). (H&E, X200)

Fig. C: Testis of a MTX-treated rat showing marked degeneration (arrow) of most spermatocytes , spermatids and congestion in the testis blood vessels. (H&E, X200)

Fig. D: Testis of a rat given mulberry extract at 100 mg.kg b.wt. for 4 weeks showing normal histological structure of most seminiferous tubules, spermatozoa (SZ) were well developed and released into the lumen. (H&E, X200)

Fig. E: Testis of a MTX- treated rat given mulberry extract at 100 mg.kg b.wt. for 4 weeks showing normal histological structure of most seminiferous tubules, spermatozoa (SZ) were well developed and released into the lumen. (H&E, X200).

## DISCUSSION

Methotrexate, an immunosuppressive drug used to treat cancer, psoriasis, and rheumatic diseases, is a folic acid antagonist that binds to the enzyme dihydro-folate reductase. This inhibits synthesis of thymidylate, serine, and methionine, which disrupts synthesis of DNA, RNA, and protein and leads to cell death (French and Koren, 2003).

The present study demonstrated administration of MTX caused significant decrease in GSI. According to Moore and Dalley (1999), an increase in organ body weight ratio is an indication of inflammation while a decrease is an indication of cell constriction. Thus significant decrease in testis-body weight ratio by the administration of the MTX may be attributed to increased cell death of the germ cells which in this study is supported by the decrease in testosterone as well as FSH.

The results of these studies indicate that administration of the MTX at the dose of 20 mg/kg body weight to male rats markedly influences the levels of sex hormones. The principal observations were: First, there was a pronounced decrease in testosterone level when compared to controls; second the level of FSH was significantly less than in controls; and, finally, LH also showed significant reduction than in controls.

There were a marked distortion in the testicular seminiferous tubules following the administration of the MTX at the dose of 20 mg/kg body weight, when compared with the control. The repeated administration of the extract at doses of 100 mg/kg body weight for a period of 28 days may suggest a possible influence on the male rat fertility. Gametogenesis occurs in the seminiferous tubules while the interstitial cells secrete the testicular hormone, mainly testosterone (Prasad and Rajalakshmi, 1989). Therefore, any alteration in the seminiferous tubules as

observed in the histopathological studies will have its consequential effect on gametogenesis.

Medicinal plants, since times immemorial, have been used in virtually all cultures as a source of medicine. The widespread use of herbal remedies and healthcare preparations and obtained from commonly used traditional herbs and medicinal plants, has been traced to the occurrence of natural products with medicinal properties. The use of traditional medicine and medicinal plants in most developing countries, as a normative basis for the maintenance of good health, has been widely observed (Hoareau and Dasilva, 1999). Nearly 80% of the world populations rely on traditional medicines for primary health care, most of which involve the use of plant extracts (Sandhya *et al.*, 2006). Previous studies have shown the fertility-enhancing properties and spermatogenic effects of some plant extracts (Kamal *et al.*, 2003).

In this study, oral administration of alcoholic extract of *Morus alba* (100 mg/kg), for 28 days, increase the hormone level of testosterone, FSH and LH as compared with MTX treated group. The elevation in testosterone might be due to this extract, which altered androgen hormones synthesis of Leydig cells. Where it enhance spermatogenesis. There is no data concerning the effect of mulberry extract on fertility of rat for this reason it is very difficult to compare our results with those reported by other authors.

The present study displayed that ethanolic extracts of mulberry leaf orally administered for 28 days, effectively enhance male rat fertility and reduce the adverse effects after administration of MTX which caused significant decrease in male sex hormone levels. Meanwhile, the pathological examination demonstrated that mulberry leaf showed prevention of testis degeneration results

from MTX administration. Results of this study supported the fertility-enhancing properties of mulberry leaf extract.

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## ARABIC SUMMARY

### التأثير الوقائي لمستخلص أوراق التوت على خصوبة ذكور الجرذان المتأثرة بسمية الميثوتريكسات

إيناس عبد الحى طه طلبه

قسم الاحياء – كلية العلوم – جامعة جازان - المملكة العربية السعودية

يستخدم الميثوتريكسات على نطاق واسع في العلاج من امراض السرطان، ومرض الصدفية، والأمراض الروماتيزمية ولكن يمكن للميثوتريكسات إحداث سمية للجهاز التناسلي. يعرف عن نبات التوت باحتوائه على مضادات للأكسدة هامة. وبالتالي، فإن الدراسة الحالية تهدف للتحقيق في الآثار الوقائية لمستخلص الايثانول لاورق نبات التوت ضد السمية الإنجابية للميثوتريكسات في ذكور الجرذان. تم تقسيم عشرين من ذكور الجرذان البيضاء إلى أربع مجموعات متساوية (ن = 5)، المجموعة الاولى تمثل المجموعة الضابطة، والثانية تم معالجتها بمستخلص اوراق التوت (100مجم /كجم) من وزن الجسم، تلقت المجموعة الثالثة الميثوتريكسات (20 مجم /كجم)، واخير تلقت المجموعة الرابعة بعد أسبوع واحد من العلاج بمستخلص اوراق التوت (100مجم /كجم) جرعة من الميثوتريكسات تعادل (20 مجم /كجم) واستمر العلاج بمستخلص نبات التوت لمدة اربعة اسابيع. نتائج الدراسة تظهر أن الميثوتريكسات تسبب في انخفاض ملحوظ في أوزان مؤشر Gonadisomatic، هرمون التستوستيرون، هرمون حث الجريبات (FSH)، هرمون ملوتن (LH) في مصل الدم وبالمقارنة بالمجموعة الضابطة. انعكس التأثير الوقائي لاوراق نبات التوت تجاه السمية المستحثة بواسطة الميثوتريكسات في الزيادة لهرمونات التستوستيرون هرمون حث الجريبات (FSH)، هرمون ملوتن (LH) بالمقارنة مع تلك الموجودة في الجرذان المعالجة بالميثوتريكسات. ( $P < 0.05$ ). كشف الفحص النسيجي أيضا أنه يوجد أضرار داخل الأنابيب المنوية وضمور للأوعية الدموية و الخلايا الجرثومية وخلايا سيرتولي لوحظت في الفئران المعالجة بالميثوتريكسات في المقابل، كان تم تقادي هذه الاضرار بشكل ملحوظ في المجموعة المعالجة بمستخلص نبات التوت. في الختام، اوضحت الدراسة ان المعاملة بمستخلص اوراق التوت يخفف من المخاطر المرتبطة بالمعالجة بالميثوتريكسات ويحمي أنسجة الخصية.